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Isolation and Characterization of Infected and Uninfected Cells from Soybean Nodules[1]

ROLE OF UNINFECTED CELLS IN UREIDE SYNTHESIS

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ABSTRACT

The distribution of organelles and associated enzymes between cells containing bacteroids and uninfected cells from nodules of *Glycine max* L. Merr. cv Amsoy 71 was investigated by separation of protoplasts on a sucrose step-gradient. Infected protoplasts were much larger, irregular in shape, and more dense than uninfected protoplasts. The peroxisomal enzymes, uricase and catalase, were present at much higher specific activity in the uninfected cell fraction. Allantoinase, an enzyme of the endoplasmic reticulum, had a greater specific activity in the uninfected cell fraction. Several enzymes whose products are required for purine biosynthesis, including phosphoglycerate dehydrogenase, aspartate aminotransferase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase, exhibited a higher specific activity in the uninfected cell fraction. Isozymes of aspartate aminotransferase were separated on native gels and located by an activity stain. The soluble isozyme was predominantly found in the uninfected cell fraction. These data suggest that peroxisomes, containing uricase and catalase for conversion of uric acid to allantoin, are present only in the uninfected cells of soybean nodules. The uninfected cells also appear to be the site of the allantoinase reaction.

The ureides allantoin and allantoic acid are the major nitrogenous compounds transported in the xylem sap of soybeans (14, 15). Labeling studies have indicated that the high levels of purines synthesized in the nodule are subsequently degraded to ureides (1). The role of both cellular and subcellular compartmentation in this process has been implicated in several recent reports. Uricase and catalase were localized in peroxisomes, allantoinase in the ER, and xanthine dehydrogenase in the cytosol by fractionation of a total nodule tissue extract on sucrose density gradients (10). By means of electron microscopy, Newcomb and Tandon (16) observed that a marked enlargement of peroxisomes and proliferation of SER during nodule development occurred only in the uninfected cells of nodules, and suggested that these cells serve a role in ureide production. In further cell fractionation studies (5), several enzymes involved in ammonia assimilation and purine biosynthesis were localized in the plastid. These included P-ribosyl amidotransferase, P-glycerate dehydrogenase, serine hydroxymethyl transferase, methylene tetrahydrofolate dehydrogenase, one isozyme of aspartate aminotransferase, glutamate synthase, and triose-P isomerase.

Separation of infected and uninfected nodule cells is necessary to determine the distribution of enzymes and organelles involved in ureide production. We have separated protoplasts on a sucrose step-gradient and assayed the uninfected and infected cell fractions for enzymes involved in purine synthesis and degradation.

MATERIALS AND METHODS

Seeds of *Glycine max* L. Merr. cv Amsoy 71 were inoculated with *Rhizobium japonicum* strain 3I1b 110 obtained from D. Weber, United States Department of Agriculture, Beltsville, MD. Plants were grown in a growth chamber in perlite and watered daily with nitrogen-free nutrient solution. For protoplast isolation, 6 g of nodules from plants 50 d old were finely sliced with a razor blade in a Petri dish containing about 5 ml of 1-B5 tissue culture medium of Gamborg (8). This medium was removed with a pipet, and the tissue was rinsed four times with fresh 1-B5 medium to remove broken cell contents. Then 11 ml of 1-B5 medium and an enzyme solution containing 200 mg Cellulysin (Calbiochem), 100 mg hemicellulase (Sigma), and 0.2 ml pectinase (Sigma), 1 g sorbitol, and 9 ml H_2O were added. The dish was shaken at 25 rpm for 1.5 to 2.5 h at 25°C. The brei was passed through 100-μm nylon mesh. Approximately 16 ml of the protoplast suspension were very gently layered onto the following step gradient, prepared immediately before use, in a 30-ml nitrocellulose tube: 3 ml of 60% sucrose, 7 ml of 40% sucrose, and 10 ml of a B5 wash medium (8). All sucrose solutions were prepared in B5 medium. The gradient was centrifuged at very low speed (about 30*g*) in a clinical swinging bucket centrifuge for 3 to 5 min, when two bands at the interfaces could be seen. One-ml fractions were collected from the top of the gradient using an ISCO model 185 Density Gradient Fractionator.

Uricase, allantoinase, and β-hydroxybutyrate dehydrogenase were assayed as previously reported (10). Catalase was determined by the decrease in *A* at 240 nm (13). Triose-P isomerase was determined by coupling to α-glycerophosphate dehydrogenase (2). Aspartate aminotransferase was assayed by coupling to malate dehydrogenase (4). P-ribosyl amidotransferase was assayed by PRPP[2]-dependent deamidation of [^{14}C]glutamine (11), which was separated from labeled glutamate by ion exchange (18). P-glycerate dehydrogenase was assayed by P-hydroxypyruvate-dependent oxidation of NADH at 340 nm (5). The assay for glucose-6-P dehydrogenase contained 2 mM glucose-6-P, 0.2 mM NADP, and 20 mM Tricine (pH 7.8). The increase in *A* at 340 nm was

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[2] Abbreviations: PRPP, 5-phospho-α-D-ribose 1-pyrophosphate; PAGE, polyacrylamide gel electrophoresis; Fast Violet B, 6-benzamide-4-methoxy-*m*-toluidine, diazonium chloride.

measured. The assay for 6-P-gluconate dehydrogenase was identical, except for substitution of 2 mM 6-P-gluconate for glucose-6-P. Lactate dehydrogenase was assayed at 340 nm in 0.15 mM NADH, 2 mM pyruvate or hydroxypyruvate, and 50 mM phosphate buffer (pH 7.5). The assay for malate dehydrogenase contained 0.2 mM NADH, 3.3 mM oxaloacetate, and 20 mM Tricine (pH 7.8). The decrease in *A* at 340 nm was measured. Native PAGE was performed according to Laemmli (12), omitting SDS. The gels were stained for aspartate aminotranferase activity with Fast Violet B salt (Sigma) (19). Protein was determined by a modified Lowry procedure (3).

RESULTS

Although protoplasts have been previously isolated from leguminous nodules (6, 23), only infected cells were obtained. In these studies, we have separated two types of protoplasts (Fig. 1). The larger protoplasts were irregular in shape and had a granular appearance, similar to previously isolated infected protoplasts (6, 19). Staining with Calcofluor White indicated no cell wall material remained. The second type of protoplasts were much smaller and spherical, and were enzymically characterized as being uninfected cells (Table I). These smaller spherical cells did not come from the cortex, since control experiments using only infected tissue yielded both small spherical and infected protoplasts, and no protoplasts were obtained from cortex tissue after 3 h digestion (data not shown). The smaller uninfected protoplasts proved to be more fragile and were often obtained in lower yield than the infected protoplasts (Table I). The infected protoplasts had a very high density, probably due to the large number of bacteroids per cell, and rapidly pelleted through 50% sucrose even at low centrifugal force. Attempts to separate the two types of protoplasts by flotation were unsuccessful, and the density of the dextran gradients of Edwards *et al.* (7) were not high enough to band the infected protoplasts. The sucrose step-gradient specified above was designed to band the uninfected protoplasts at the upper interface and the infected protoplasts at the lower interface. It was essential to minimize handling of the protoplasts, because the uninfected cells were easily broken by contact with the larger infected cells and the high sucrose concentration necessary for

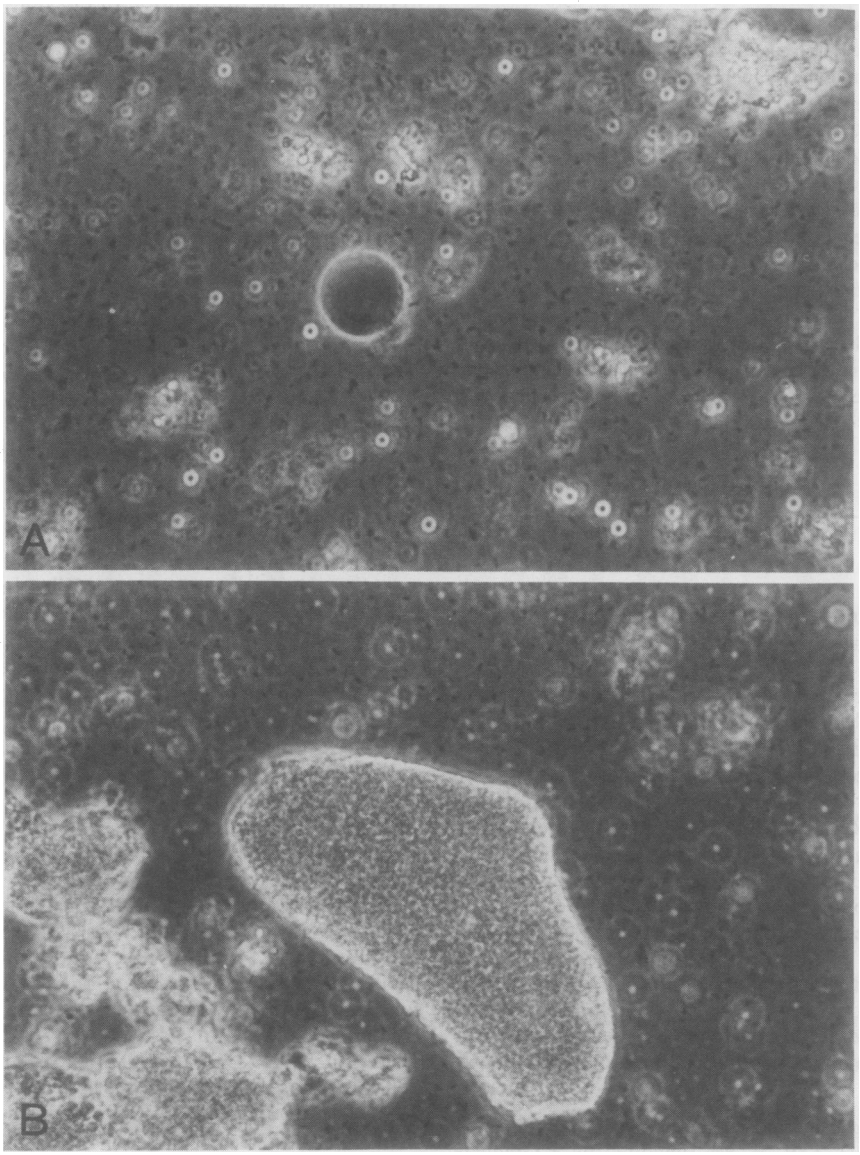

FIG. 1. Light micrographs of a crude protoplast suspension before separation on the step-gradient. Background debris from broken cells was removed in the upper layer of the gradient. Magnification: × 25. A, Uninfected cell; size ranged from 20 to 50 μm in diameter. B, Infected cell; size ranged from 100 to 200 μm in diameter.

Table I. *Enzyme Activity from a Step-Gradient for Separation of Protoplasts*

The total uninfected cell or infected cell fractions were pooled separately from a sucrose step-gradient as described in "Materials and Methods."

	Uninfected Cell Fraction		Infected Cell Fraction	
	total $nmol \cdot min^{-1}$	$nmol \cdot min^{-1} \cdot mg^{-1}$ *protein*	*total* $nmol \cdot min^{-1}$	$nmol \cdot min^{-1} \cdot mg^{-1}$ *protein*
Uricase	20	51	4.5	5.5
β-Hydroxybutyrate dehydrogenase	0.2	0.6	3.5	4.3
P-glycerate dehydrogenase	25	64	20	24
Asparate aminotransferase	20	51	18	22
Total protein ($mg \cdot fraction^{-1}$)	0.39		0.82	

Table II. *Specific Activity of Enzymes Involved in Ureide Formation*

β-Hydroxybutyrate dehydrogenase was used as a marker enzyme for infected cells and Triton X-100 was added for its assay.

	Uninfected Cell Fraction	Infected Cell Fraction
	$nmol \cdot min^{-1} \cdot mg^{-1}$ *protein*	
Uricase	65	4.1
Catalase	35×10^3	5.0×10^3
Allantoinase	9.0	3.6
β-Hydroxybutyrate dehydrogenase	1.5	6.0

Table III. *Specific Activity of Enzymes Involved in Purine Synthesis and Energy Metabolism*

Triton X-100 was not present during assays.

	Uninfected Cell Fraction	Infected Cell Fraction
	$nmol \cdot min^{-1} \cdot mg^{-1}$ *protein*	
P-Glycerate dehydrogenase	58	27
Aspartate aminotransferase	79	30
6-P-Gluconate dehydrogenase	4.4	1.1
Glucose-6-P dehydrogenase	3.7	1.1
Triose-P isomerase	150	140
Malate dehydrogenase	7.2	3.5
Lactate dehydrogenase	7.0	5.4

separation. Yields of each protoplast type were low, ranging from 0.5 to 3.0 mg protein, depending on the amount of tissue (from 1–6 g nodules) used and incubation time. Longer periods of incubation with the digestive enzymes yielded fewer uninfected cells but more infected cells, and often resulted in higher levels of cross-contamination. Only by using the 1-B5 and B5 media of Gamborg (8) were substantial quantities of uninfected protoplasts obtained. More uninfected protoplasts were obtained from nodules of 40 to 50-d-old plants than from nodules of younger plants.

Enzyme analysis from a representative separation of infected and uninfected protoplasts is presented in Table I. Uricase was the marker enzyme for peroxisomes (10) and β-hydroxybutyrate dehydrogenase was the bacteroid marker enzyme. The lower protoplast fraction at the 60% sucrose interface contained 95% of the β-hydroxybutyrate dehydrogenase activity, and was therefore designated the infected cell fraction. The upper protoplast fraction at the 40% sucrose interface contained only 5% of the β-hydroxybutyrate dehydrogenase activity and was designated the uninfected cell fraction. The majority of the uricase activity (82%) was found in the uninfected cell fraction. Data are also shown for P-glycerate dehydrogenase and aspartate aminotransferase, two enzymes which have been localized in the plastid fraction of the nodules (5) and are presumably involved in purine biosynthesis. The specific activities of these two enzymes were 2 to 3 times higher in the uninfected cell fraction, but about half of the total activity was also present in the infected cell fraction.

On the basis of total mg protein, the yield of uninfected protoplasts was about half that of infected cell protoplasts. The enzymic specific activities were quite reproducible between experiments in which the yields of each cell type might vary. Table I contains both total units of enzyme activity and specific activity for comparison. It was not feasible to perform cell counts by packed cell volume due to the low yield of purified protoplasts, nor could bacteroids be separated from cellular membranes of the infected cell fraction. Triton-X-100 was present only in the assay for β-hydroxybutyrate dehydrogenase, in order to break the bacteroid membranes (10). Addition of low concentration of detergent (0.01%) did not increase any of the other enzyme activities, indicating cellular membranes were probably broken during dilution into the assay, and were not a barrier to detection of enzyme activity.

Average specific activities from 25 different step-gradients are presented in Table II. Uricase and catalase, the peroxisomal enzymes (10), were predominantly in the uninfected cell fraction. Allantoinase, located in the ER (10), was also mostly in the uninfected cell fraction. These data confirm the hypothesis that peroxisomes and the ER which contain the enzymes that catalyze the final steps in ureide formation are located in the uninfected cells (16).

Average specific activities for several of the metabolic enzymes which are involved in purine biosynthesis are given in Table III. Inasmuch as none of these assays contained detergent, it may be assumed that the bacteroids made no contribution to the activities as measured. P-glycerate dehydrogenase is a plastid enzyme, probably involved in synthesis of serine, which is required for purine biosynthesis (5). Its specific activity was twice as high in the uninfected cell fraction as in the infected cell fraction.

Specific activity of aspartate aminotransferase (Table III) was 2 to 3 times higher in the uninfected cell fraction. Isozymes of aspartate aminotransferase in the nodule have been well characterized (20, 22) and one isozyme of aspartate aminotransferase in the soybean nodule has recently been localized in the plastid (5), where aspartate is presumably required for purine synthesis. The presence of different isozymes of aspartate aminotransferase in peroxisomes, mitochondria, and chloroplasts of leaves has been previously reported (19).

Protoplast fractions were subjected to native PAGE (12) and

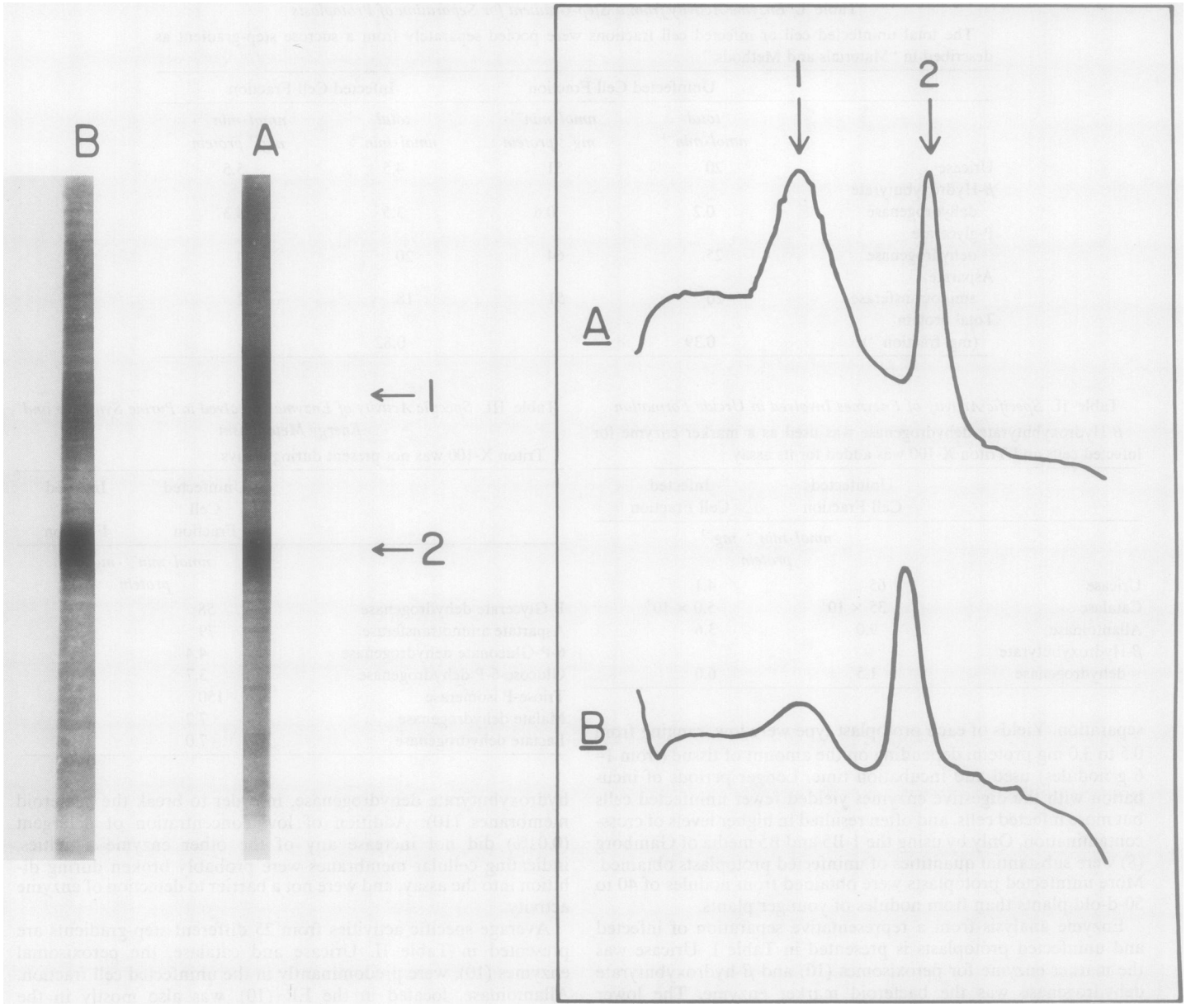

FIG. 2. Isozymes of aspartate aminotransferase separated by native PAGE. Activity was stained with Fast Violet B. Band 1 is the soluble isozyme; band 2 is the plastid isozyme (5). (A), Uninfected cell fraction; (B), Infected cell fraction.

stained for aspartate aminotransferase activity (Fig. 2). Significantly more of the soluble aspartate aminotransferase isozyme (Fig. 2, band 1) was located in the uninfected cell fraction (gel A). Approximately equal amounts of the plastid isozyme (Fig. 2, band 2) were observed in both cell fractions. At least part of the soluble activity observed in the infected cell fraction (gel B) was due to contamination by uninfected cells (indicated by uricase activity).

Glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase are enzymes of the oxidative pentose phosphate pathway, of which one product is ribulose-5-P, which is converted to ribose-5-P by ribose-P isomerase. Ribose-5-P is used in the production of PRPP for use in purine biosynthesis. Therefore, high levels of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase would be expected in cells synthesizing large amounts of purines. Indeed, the specific activity of both of these enzymes in the uninfected cell fraction was about four times the specific activity in the infected cell fraction (Table III). Starch is the major source of glucose-6-P in most plant cells (17). We have noted starch granules in the plastids of only the uninfected cells in the electron micrographs of Newcomb and Tandon (16).

Triose-P isomerase activity was present in both the cytosol and plastid fractions (5). The specific activity of this enzyme was essentially equal in both uninfected and infected cell fractions. The specific activity of malate dehydrogenase in the uninfected cell fraction was about twice that in the infected cell fraction (Table III). While nodule peroxisomes contained no hydroxypyruvate dehydrogenase (data not shown), lactate dehydrogenase was present in the cytosol, and utilized pyruvate, hydroxypyruvate, or glyoxylate as a substrate (data not shown). The specific activity of lactate dehydrogenase was only slightly higher in the uninfected cell fraction (Table III).

Results of assays for P-ribosyl amidotransferase (the enzyme catalyzing the first committed step of purine biosynthesis) were inconclusive. Both uninfected and infected cell fractions contained very low levels of this enzyme activity (data not shown). Although this enzyme has been measured in nodule extracts, the specific activity was quite low compared with uricase (21), for example, and one would not expect to detect activity in the low yields of

pure protoplasts obtained in our experiments. PRPP synthetase was also not detectable. It will probably only be possible to localize these enzymes in protoplasts by labeling studies. P-ribosyl amidotransferase has been localized intracellularly in the plastid (5).

DISCUSSION

Several intracellular compartments of nodule cells participate in purine synthesis and degradation to ureides, including plastids, peroxisomes, the ER, and the cytosol (5, 10). Most of the activity of the peroxisomal enzymes uricase and catalase was associated with the uninfected cell fraction. Allantoinase, which has been localized in the ER (10), also had a much greater specific activity in the uninfected cell fraction. All of these data support the previous report (16), based on electron micrographs, that peroxisomes are found predominantly in the uninfected cells, where SER also proliferates.

Enzymes of purine biosynthesis have been localized in the plastids (5), which are observed in both cell types in the soybean nodule (16). Purine synthesis might therefore occur primarily in only infected cells, only in noninfected cells, or in both cell types. Several of the enzymes whose products are required for purine synthesis, including P-glycerate dehydrogenase, aspartate aminotransferase, 6-P-gluconate dehydrogenase, and glucose 6-P dehydrogenase, were present at much higher levels in uninfected cells, and the soluble isozyme of aspartate aminotransferase was predominantly found in the uninfected cell fraction. However, the possibility that purine synthesis occurs in the infected cells as well cannot be excluded on the basis of enzyme distribution alone. If purine synthesis does occur primarily in the uninfected cells, amino acids are probably transported there from the infected cells. However, if purine synthesis occurs in the infected cells, a purine intermediate might be transported. These and other questions may be resolved by labeling studies using purified protoplasts.

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LITERATURE CITED

1. ATKINS CA, R RAINBIRD, JS PATE 1980 Evidence for a purine pathway of ureide synthesis in N_2-fixing nodules of cowpea. Z Pflanzenphysiol 97: 249–260
2. BEISENHERZ G 1955 Triosephosphate isomerase from calf muscle. Methods Enzymol 1: 387–391
3. BENSADOUN A, D WEINSTEIN 1976 Assay of proteins in the presence of interfering materials. Anal Biochem 70: 241–250
4. BERGMEYER HU, E BERNT 1963 Glutamate-oxaloacetate transaminase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis. Academic Press, New York, pp 837–844
5. BOLAND MJ, JF HANKS, PHS REYNOLDS, DG BLEVINS, NE TOLBERT, KR SCHUBERT 1982 Subcellular organization of ureide biogenesis from glycolytic intermediates and ammonium in nitrogen-fixing soybean nodules. Planta 155: 45–51
6. DAVEY MR, EC COCKING, E BUSH 1973 Isolation of legume root nodule protoplasts. Nature 244: 460–461
7. EDWARDS GE, R MCLILLEY, S CRAIG, MD HATCH 1979 Isolation of intact and functional chloroplasts from mesophyll and bundle sheath protoplasts of the C_4 plant *Panicum miliaceum*. Plant Physiol 63: 821–827
8. GAMBORG OL 1975 Callus and Cell Culture. *In* OL Gamborg, LR Wetter, ed, Plant Tissue Culture Methods. National Research Council of Canada, Saskatoon, Saskatchewan, pp 1–11
9. HANKS JF, NE TOLBERT 1982 Ureide metabolism in protoplasts from infected and uninfected cells of soybean nodules. Plant Physiol 69: s-117
10. HANKS JF, NE TOLBERT, KR SCHUBERT 1981 Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules. Plant Physiol 68: 65–69
11. HOLMES EW, JA MCDONALD, JM MCCORD, JB WYANGAARDEN, WN KELLEY 1973 Human glutamine phosphoribosylpyrophosphate amidotransferase. J Biol Chem 248: 144–150
12. LAEMMLI VK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
13. LUCK H 1965 Catalase. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Ed 2. Academic Press, New York, pp 885–894
14. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977 Incorporation of ^{15}N into allantoin in nodulated soybean plants supplied with ^{15}N. Plant Cell Physiol 18: 459–462
15. MCCLURE PR, DW ISRAEL 1979 Transport of nitrogen in the xylem of soybean plants. Plant Physiol 64: 411–416
16. NEWCOMB EH, SR TANDON 1981 Uninfected cells of soybean root nodules: ultrastructure suggest key role in ureide production. Science 212: 1394–1396
17. PREISS J, C LEVI 1980 Starch biosynthesis and degradation. *In* J Preiss, ed, The Biochemistry of Plants, Vol 3. Academic Press, New York, pp 371–417
18. PRUSINER S, L MILNER 1970 Rapid radioactive assay for glutamine synthetase, glutaminase, asparagine synthetase, and asparaginase. Anal Biochem 37: 429–438
19. REHFELD DW, NE TOLBERT 1972 Aminotransferase in peroxisomes from spinach leaves. J Biol Chem 247: 4803–4811
20. REYNOLDS PHS, KJF FARNDEN 1979 The involvement of aspartate aminotransferases in ammonium assimilation in lupin nodules. Phytochemistry 18: 1625–1630
21. REYNOLDS PHS, MJ BOLAND, DG BLEVINS, KR SCHUBERT, DD RANDALL 1982 Enzymes of amide and ureide biogenesis in developing soybean nodules. Plant Physiol 69: 1334–1338
22. RYAN E, F BODLEY, PF FOLTRELL 1972 Purification and characterization of aspartate aminotransferases from soybean root nodules and *Rhizobium japonicum*. Phytochemistry 11: 957–963
23. WOO KC, WJ BROUGHTON 1979 Isolation and metabolism of *Vigna unguiculata* root nodule protoplasts. Planta 145: 487–495